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Poliovirus type 1/type 3 antigenic hybrid virus constructed *in vitro* elicits type 1 and type 3 neutralizing antibodies in rabbits and monkeys

(polio neutralizing antigenicity sites/divalent polio vaccine)

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ABSTRACT **Poliovirus exists as three stable serotypes (PV-1, PV-2, and PV-3). These viruses display three antigenic sites each, designated N-AgI, N-AgII, and N-AgIII. When mice are immunized with poliovirus, N-AgI is the major neutralization antigenic site for PV-3, whereas N-AgII and N-AgIII are immunodominant over N-AgI for PV-1. To study the relationship between structure and antigenicity, a hybrid virus was constructed in which N-AgI of PV-1 was replaced by N-AgI of PV-3. PV-3- and PV-1-specific antisera, including those elicited by PV-3 in primates, neutralized the hybrid virus. Injection of the hybrid virus into rabbits or into primates resulted in the production of antisera that neutralized both PV-1 and PV-3. The data show that sequence replacement at N-AgI of poliovirus is compatible with viral proliferation, an observation useful for the development of multivalent picornavirus vaccines.**

Poliovirus causes poliomyelitis, an acute disease of the central nervous system that leads to paralysis or death. Although two excellent poliovirus vaccines (inactivated virus and live attenuated virus; see refs. 1 and 2 and references therein) are used in developed countries, poliomyelitis remains a serious health problem worldwide. The total eradication of circulating poliovirus is desirable and, indeed, is a goal of international health organizations. However, this requires continuing research on the physical and biological properties of the poliovirion, its pathway of infection, and the response of the immune system to virus infection. We report here the construction of a hybrid poliovirus that carries neutralization epitopes of two different serotypes of poliovirus.‖

Poliovirus is a picornavirus whose naked capsid is composed of 60 copies each of viral polypeptides VP1 and VP3, 68 or 69 copies of VP2 and VP4, and 1 or 2 copies of VP0, the precursor to VP2 and VP4 (3). The polypeptide shell encloses a single-stranded positive-sense RNA genome about 7500 nucleotides long (4). The genome sequences of representatives of all three serotypes of poliovirus (including the Sabin vaccine strains) have been determined and found to be closely related (4–9).

Each of the three poliovirus serotypes is defined by its ability to elicit neutralizing antibodies that are not capable of neutralizing the other two serotypes. Work in several laboratories has led to the discovery of three independent neutralizing antigenic sites (N-Ags) and their neutralization epitopes of poliovirus (see refs. 10–16 for further references). The solution of the three-dimensional structure of the poliovirion has subsequently permitted the mapping of the N-Ags to specific surface regions of the viral particle (17). Interestingly, the antigenic structure of poliovirus was found to be nearly identical to that of human rhinovirus 14 (18).

Of the three N-Ags, N-AgI is a continuous sequence of amino acids mapping to positions 90–105 from the N terminus of capsid protein VP1 (Fig. 1*C*). Amino acids 96–104 of VP1 are located at the apex of the viral particle and exist as a loop that is particularly well exposed (17). N-AgI of PV-1 is recognized by two exceptional neutralizing monoclonal antibodies (N-mAbs C2 and 95) even after denaturation of capsid protein VP1 (20, 21). The other two neutralization antigenic sites (N-AgII and N-AgIII) are discontinuous in nature. N-AgII is composed of amino acids in the region 220–222 from the N terminus of VP1 plus amino acids 164–175 of VP2. N-AgIII is composed of amino acids 286–290 from the N terminus of VP1 plus amino acids 58–60 and 71–79 from the N terminus of VP3.

A paradox appeared during the early studies of the antigenicity of poliovirus. PV-1 elicited N-mAbs in Balb/c mice that recognized N-AgII and N-AgIII but not N-AgI. On the other hand, the N-mAbs isolated from Balb/c mice inoculated with PV-3 exclusively recognized N-AgI (see the discussion by Wimmer *et al.* in ref. 22). This paradox was solved in that the phenomenon of different immunodominance of N-Ags of poliovirus types was found predominantly when specific strains of inbred mice were used for the monoclonal antibody production. In outbred mammals the immunodominance of a particular N-Ag over others is much less apparent (23). Considering the close relationship of the poliovirus serotypes in regard to primary structure, the different responses of immune systems to the different serotypes remain an unexplained observation.

The unique structure of N-AgI within the poliovirion and its peculiar interaction with the immune system makes it an ideal target for *in vitro* induced mutagenesis. It is of interest to investigate what amino acids in this loop determine type

Abbreviations: PV-1(M), PV-2(L), PV-2(MEF1), and PV-3(L), polioviruses type 1 (Mahoney), type 2 (Lansing), type 2 (strain MEF1), and type 3 (Leon), respectively; N-Ags, neutralizing antigenic sites; N-AgI, N-AgII, and N-AgIII, neutralizing antigenic sites 1, 2, and 3, respectively; N-mAb, neutralizing monoclonal antibody; $TCID_{50}$, dose required to infect 50% of cells in tissue culture. The nomenclature suggested by Rueckert and Wimmer (31) is used for virus-specific polypeptides in which capsid proteins VP4, VP2, VP3, and VP1 also are called 1A, 1B, 1C, and 1D, respectively.

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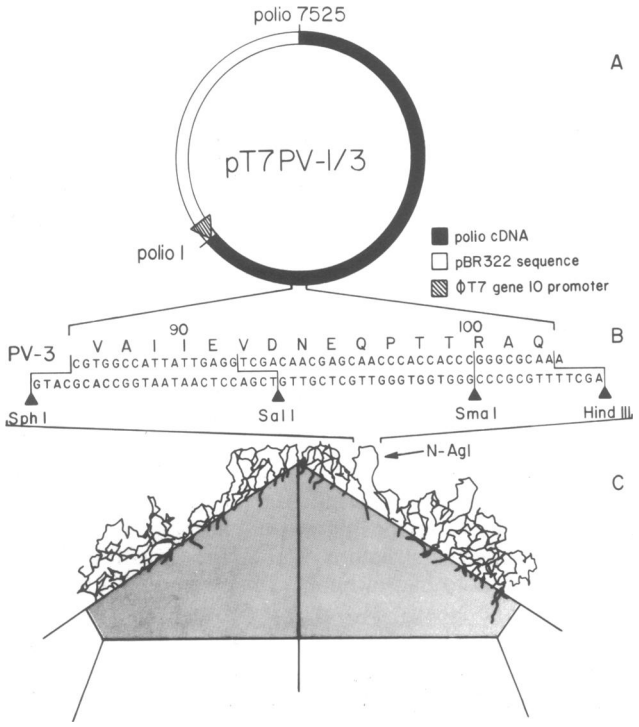

FIG. 1. Schematic representation of the strategy to construct a poliovirus type 1/type 3 hybrid virus. (*A*) Plasmid pT7PV-1/3, a derivative of transcription vector pT7PV1-5 (28), was constructed as described in the text. It contains the phage T7 (ϕT7) promoter for T7 RNA polymerase and the cDNA sequence of PV-1(M) in which a small DNA segment encoding the amino acid sequence N-AgI was replaced with that of PV-3(L). (*B*) Sequence corresponding to a region specifying N-AgI of PV-3(L). (*C*) The loop is located near the apex of the poliovirion (17); *C* was redrawn from a figure published by Hogle *et al.* (17).

specificity and to what extent amino acids in this loop can be modified without losing viability of the virus. Such study can be the basis of the development of multivalent picornavirus vaccines.

MATERIALS AND METHODS

Oligonucleotide-Mediated Site-Specific Mutagenesis of PV-1. The construction of the mutagenesis cartridge was achieved by cloning the appropriate PV-1 fragments into phage M13mp10 (24) followed by site-directed mutagenesis of the poliovirus genome (25). Single-strand template DNA was prepared in *Escherichia coli* strain BW313, (*dut*$^-$ *ung*$^-$) (26), and its sequence was altered by mismatch oligonucleotides as described (25). Details of the protocol will be published elsewhere. Mutants were identified by restriction enzyme analysis and nucleotide sequencing.

Immunizations and Neutralization Assays. The hybrid virus was grown on monolayer HeLa cells and harvested after complete CPE (≈48 hr). Approximately 10 μg of total protein (determined by the Lowry method or by colorimetry with the brilliant blue G-250 Bio-Rad kit) in a volume of 50 μl was emulsified 1:1 with incomplete Freund's adjuvant and injected into female New Zealand White rabbits via a peripopliteal lymph node route. At the same time, ≈50 μg (total protein) was emulsified 1:1 with complete Freund's adjuvant and injected via the subcutaneous (25 μg) and intramuscular (25 μg) routes. At 10- to 14-day intervals, three boosters with 50 μg of CsCl-purified virus emulsified 1:1 with incomplete Freund's adjuvant were injected intramuscularly into the rabbits' gastronemeous muscle. Cynomolgus monkeys were twice injected subcutaneously with doses of hybrid virus as indicated in Table 4. Sera were withdrawn at regular intervals and tested for their ability to neutralize different strains of poliovirus.

Two different assays were used to assess the neutralizing titers of immune sera. First, plaque reduction assays were performed essentially as described by Emini *et al.* (19). Briefly, virus stocks were diluted by serial 1:10 dilution; 0.1 ml of each dilution was incubated with 0.1 ml of undiluted antiserum for 1 hr at room temperature. Each mixture was then used to infect confluent HeLa cell monolayers growing in six-well dishes (Falcon 3046). Plaques were then counted at ≈48 hr postinfection after staining with crystal violet or neutral red. Second, serial dilutions of sera were titered by their ability to neutralize a fixed dose of poliovirus (100 $TCID_{50}$; $TCID_{50}$ is the dose required to infect 50% of the cells in tissue culture).

RESULTS

We have asked the question whether the replacement of N-AgI in PV-1 strain Mahoney [PV-1(M)] with N-AgI of PV-3 strain Leon [PV-3(L)] is compatible with viral proliferation and, if so, whether the exchanged sequence will be an active determinant participating in the induction of neutralizing antibodies and in neutralization. To facilitate virtually unlimited genetic variation of N-AgI, we constructed a mutagenesis cartridge (Fig. 1*B*) in this region. We followed a strategy previously used by us to produce mutants in the genome-linked protein VPg of poliovirus (27). A synthetic double-stranded DNA segment encoding N-AgI of PV-3 (Fig. 1*B*) was inserted between a naturally occurring *Sph* I restriction site (nucleotide number 2732 of PV-1) and a newly generated *Hin*dIII site (nucleotide number 2786) replacing the existing PV-1 sequences. All nucleotide changes necessary for cloning and insertion of the mutagenesis cartridge preserved the expected amino acid sequence of the virus. After genetic manipulation, the poliovirus-specific cDNA fragment was engineered into a vector containing the phage T7 promoter (Fig. 1*A*) shown previously by us to yield highly infectious poliovirus RNA after transcription by T7 RNA polymerase (28). Viral RNA was prepared *in vitro* and used to transfect HeLa cells. Wild-type virus or mutants containing amino acid replacements in region 88-102 of VP1 were isolated 36 hr after transfection and plaque-purified for further analyses. All constructs generated prior to transfections were analyzed by primer extension sequencing techniques (29). Similarly, genomic RNA of mutant virus was sequenced to confirm the presence of the nucleotide replacements.

Transfection of HeLa cells with run-off transcripts of pT7PV-1/3 yielded virus whose genomic RNA had the expected PV-1/PV-3 hybrid sequence (unpublished data). We will call this virus W1/3-1D-1, following a nomenclature suggested by Bernstein *et al.* (30); 1D denotes capsid polypeptide VP1 (31). At 37°C this hybrid virus had a small plaque phenotype, and virus yield in standard one-step growth experiments was <10% relative to wild-type PV-1(M) virus (Fig. 2).

The amino acid differences in N-AgI between strains of poliovirus and neutralization escape mutants are shown in Table 1. It is apparent that this region exhibits high interserotypic amino acid diversity. Nine amino acid differences exist between N-AgI of PV-1(M) and PV-3(L), many of which are changes in charge or size. Similarly, mutations leading to neutralization resistance involved a wide variety of different amino acid exchanges. Therefore, this region is unlikely to play an essential role in the folding pathway of the capsid precursors (aggregation of capsid precursor P1 and its proteolytic processing products VP0, VP3, and VP1) or in encapsidation, or else the numerous mutations leading to

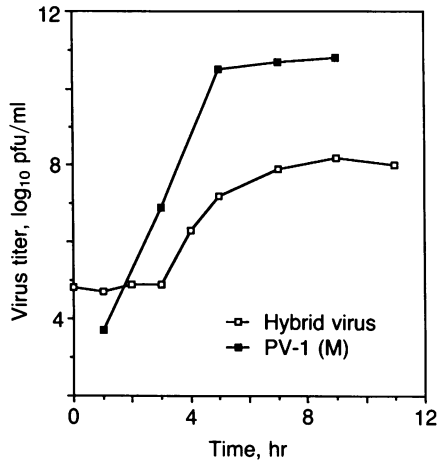

FIG. 2. One-step growth curve comparing replication of the hybrid virus with that of PV-1(M). Confluent HeLa cell monolayers growing in 60-mm tissue culture plates (Falcon 3001) were infected at a multiplicity of infection of 20 in a volume of 0.3 ml. Virus was adsorbed for 30 min at room temperature; then prewarmed medium was added, and cells were placed at 37°C in 95% air/5% CO_2. At the indicated time points, medium was aspirated, and the cells were washed twice with phosphate-buffered saline and then scraped from the plate into a 1.5-ml tube. Any remaining cells were collected by washing the plate with 1.0 ml of phosphate-buffered saline and combining this wash with the cells. Cells were pelleted and resuspended in 100 μl of buffer (0.1 M NaCl/0.1 M Tris/0.0015 M $MgCl_2$, pH 7.35) and lysed by freezing and thawing three times. Nuclei were pelleted by centrifugation, and the virus containing supernatant was collected and titered by plaque assay as described.

neutralization resistance would not be tolerable. This is underscored by our observation that the hybrid virus is viable although it is impaired in growth efficiency. The viability of the hybrid virus obtained from *in vitro* recombinant DNA is in contrast to a cDNA construct in which a much larger region of VP1 of PV-3 (Sabin) was engineered into PV-1 (Sabin). This cDNA construct did not yield virus upon transfection of HeLa cells (32). Replacement of the entire capsid region of type 1 poliovirus with that of type 3 poliovirus, on the other hand, has been reported to yield viable virus (33).

Table 2. Serological characterization of the hybrid virus with polyclonal serotype-specific hyperimmune sera

Serum	Virus titer, $\log_{10}$ pfu·ml^{-1}		
	PV-1	PV-3	PV-1/PV-3 hybrid
Monkey preimmune	8.9	8.7	8.8
Monkey anti-PV-3	9.0	<2.0	3.4
Rabbit anti-PV-1	<0.3	7.8	<0.3
Horse anti-PV-3	9.0	<0.3	<0.3

Virus titers were determined by the standard plaque-reduction assays (19).

The antigenic properties of the type 1/type 3 hybrid virus were analyzed by using polyclonal and monoclonal antisera. The serotype-specific hyperimmune sera effectively neutralized the homologous serotype virus, resulting in a reduction of the titer by >8 $\log_{10}$ (Table 2). The intertypic hybrid virus was neutralized by anti-PV-1(M) serum to the same extent as PV-1(M), an observation suggesting that the replacement of N-AgI in W1/3-1D-1 did not influence the accessibility or activity of N-AgII and N-AgIII. The hybrid virus was also neutralized significantly with monkey anti-PV-3(L), although the reduction of the plaque titer amounted to only ≈5 $\log_{10}$. As expected, the hybrid virus was not neutralized by N-mAb 95, an antibody that specifically recognizes N-AgI in PV-1(M) (21), but it was neutralized by N-mAbs 175, 194, and 204,

Table 1. Amino acid sequences of the region spanning N-AgI of various poliovirus types and strains

	90 95 100 105
PV-1(M)*	C V T I M T V D N P A S T T N K D K L F A V W
PV-1(Sabin)	- - A - I - - - - S - - - K - - - - - - - T - -
Neutralization escape mutants of PV-1	1 - - - - - - - - - - - - - - - D - - - - - - - -
	2 - - - - - - - - - - - - - - - K - - - - - - - -
PV-2(L)	- - A - I E - - - D - P - K R A S - - - S - -
PV-2(Sabin)	- - A - I E - - - D - P - K R A S R - - S - -
Neutralization escape mutants of PV-2	1 - - - - - - - - - - - - - - - P - - - - - - - -
	2 - - - - - - - - - - - - - - - - D - - - - - - -
	3 - - - - - - - - - - - - - - E - - - - - - - - -
	4 - - - - - - - - - - - - - - N - - - - - - - - -
PV-3(L)	- - A - I E - - - E Q P - - R A Q - - - - M -
PV-3(Sabin)	- - A - I E - - - E Q P - - R A Q - - - - M -
Neutralization escape mutants of PV-3	1 - - - - - - - N - - - - - - - - - - - - - - -
	2 - - - - - G - - - - - - - - - - - - - - - - -
	3 - - - - - K - - - - - - - - - - - - - - - - -
	4 - - - - - - - - - - - - I - - - - - - - - - -
	5 - - - - - - - - - - - - A - - - - - - - - - -
	6 - - - - - - - - - - - - N - - - - - - - - - -
	7 - - - - - - - - - - - - - N - - - - - - - - -
	8 - - - - - - - - - - - - - I - - - - - - - - -
	9 - - - - - - - - - - - - - - Q - - - - - - - -
	10 - - - - - - - - - - - - - - W - - - - - - - -
	11 - - - - - - - - - - - - - - - T - - - - - - -
	12 - - - - - - - - - - - - - - - V - - - - - - -
	13 - - - - - - - - - - - - - - - - L - - - - - -
	14 - - - - - - - - - - - - - - - - P - - - - - -
	15 - - - - - - - - - - - - - - - - R - - - - - -
	16 - - - - - - - - - - - - - - - - H - - - - - -

*Amino acid sequence is given in the single-letter code and is numbered according to the sequence of VP1 of PV-1(M). The numbering may be slightly different in VP1 polypeptides of other serotypes because of deletions or insertions. Data are compiled from refs. 10–16.

Table 3. Serological characterization of the hybrid virus with neutralizing N-mAb

Virus	Titer of N-mAb specific for N-AgI*			
	95†	175‡	194‡	204‡
PV-1(M)	32	<2	<1	<1
PV-3(L)	<4	1024	1024	>8192
PV-1/PV-3 hybrid W1/3-1D-1	<4	8192	256	>8192

*Inverse of the dilution of antibody sufficient to neutralize 100 $TCID_{50}$.
†Anti-PV-1(M) N-mAb 95 as described in ref. 21.
‡Anti-PV-3(L) N-mAbs as described in ref. 11.

three antibodies specific for N-AgI of PV-3(L) (11, 14) (Table 3). Moreover, two N-mAbs specific for N-AgII (N-mAb H9) and N-AgIII (N-mAb D3) of PV-1(M) (13, 15) neutralized the hybrid virus (data not shown).

The observation that hybrid virus W1/3-1D-1 expresses type 1 and type 3-specific neutralization determinants prompted us to investigate whether it could also elicit type 1- and type 3-specific antibodies upon injection into rabbits. The sera obtained from four (of four) rabbits neutralized PV-1(M), PV-3(L), and the hybrid virus but had no significant effect on the infectivity of PV-2 strain Lansing [PV-2(L)] (Fig. 3).

W1/3-1D-1 virus was also injected subcutaneously into cynomolgus monkeys, and each animal's immune response was measured. Immunization with 10^4 plaque-forming units (pfu) of hybrid virus followed by a boost of 10^7 pfu 4 weeks later showed significant titers of neutralizing antibodies against PV-1(M) and PV-1/PV-3 hybrid virus, but only a weak titer against PV-3(L) virus (Table 4). No neutralizing activity was observed against PV-2 strain MEF1 [PV-2(MEF1)] poliovirus. However, when the experiment was carried out in similar manner but with 10^7 pfu in the primary injection, significant neutralizing titers were observed against PV-1(M), PV-3(L), and the PV-1/PV-3 hybrid virus.

Table 4. Immunization of monkeys with hybrid virus

Virus	Serum titer of samples 1–6, 1/dilution neutralizing $TCID_{50}$					
	1	2	3	4	5	6
	Experiment 1 (monkey 2269)*					
PV-1(M)	<4	<4	16	64	64	1024
PV-3(L)	<4	<4	<4	<4	<4	16
PV-1/3 hybrid	<4	<4	16	64	64	4096
PV-2(MEF1)	<4	<4	<4	<4	<4	<4
	Experiment 2 (monkey 2270)†					
PV-1(M)	<4	16	1024	1024	1024	1024
PV-3(L)	<4	16	64	64	64	256
PV-1/3 hybrid	<4	64	1024	1024	1024	1024
PV-2(MEF1)	<4	<4	<4	<4	<4	<4

*The animal was inoculated subcutaneously with 10^4 pfu of PV-1/PV-3 hybrid virus (W1/3-1D-1) and given a booster after 4 weeks with 10^7 pfu of hybrid virus by the same route. Serum samples were withdrawn upon the first injection (sample 1) and then in weekly intervals (samples 2–5). Ten days after the booster injection, the final sample (6) was withdrawn.
†Immunization schedule as in experiment 1 except that the first inoculation was with 10^7 pfu of hybrid virus.

DISCUSSION

We have shown that a hybrid virus carrying neutralization antigenic sites of two different serotypes of poliovirus can be neutralized by corresponding type-specific neutralizing antibodies and can elicit the corresponding type-specific antibodies. These observations demonstrate two important points (see also ref. 34). First, in the context of the PV-1(M) sequences, the PV-3(L) insert assumes a conformation that allows binding of and neutralization with type 3-specific antibodies. Thus, the hybrid virus allows an assay for the type 3-specific neutralization epitopes of N-AgI independently of other antigenic sites of the poliovirion. Second, the type 3-specific loop in PV-3(L) and also in the PV-1/PV-3 hybrid virus is recognized by the primate immune system.

The fact that the neutralization antigenic site of one poliovirus can be replaced by a sequence with numerous amino acid changes suggests that the loop comprising this antigenic region can be replaced by heterogenous sequences representing antigenic determinants of other human pathogens. Of immediate interest to us are sequences from corre-

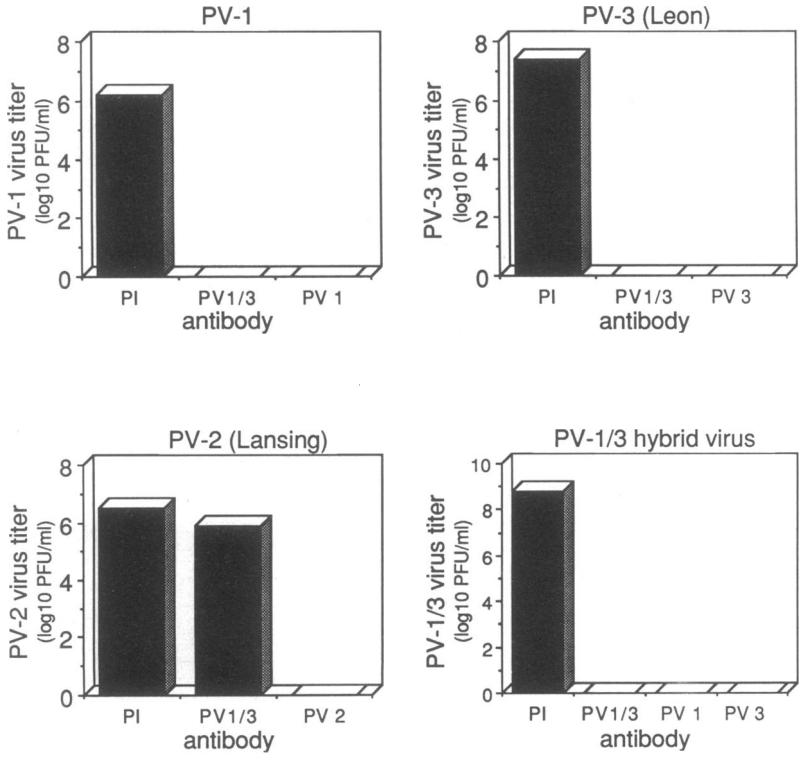

FIG. 3. Elicitation of type 1- and type 3-specific neutralizing antibodies by the PV-1/PV-3 hybrid virus (W1/3-1D-1). Standard plaque reduction assays were performed as described for Table 2. Antibody PI is rabbit preimmune serum. Antibody PV-1/PV-3 is serum from rabbits made hyperimmune to the hybrid virus, and antibodies PV-1, PV-2, and PV-3 are sera from rabbits made hyperimmune to PV-1(M), PV-2(L), and PV-3(L), respectively.

sponding regions of human rhinovirus 14 (18) and of hepatitis A virus, two other picornaviruses. The construction of a hybrid virus containing a dominant N-Ag of hepatitis A virus could lead to the development of a highly effective, oral anti-hepatitis A virus vaccine. No such vaccine against infectious hepatitis is currently commercially available. Because of the enormous serological diversity of the human rhinoviruses, a hybrid PV-1–human rhinovirus 14 virus appears to be only of academic interest.

A small but significant incidence of poliomyelitis occurs every year in the United States in spite of extensive vaccination and the virtual elimination of wild-type strains from circulation (35). Most of these cases have been classified as vaccine-associated, and it is highly likely that genetic variants of the type 2 and type 3 Sabin strains, but to a much lesser extent variants of Sabin type 1 vaccine virus, are the cause of disease in vaccine recipients or their contacts (for an extensive discussion, see refs. 2 and 35). Because of the antigenic properties of the type 1/type 3 hybrid virus reported here, we propose that genetically engineered hybrid viruses similar to W1/3-1D-1 but constructed from cDNA clones of the Sabin strain derivatives may be useful in the primary vaccination of children to reduce vaccine-associated cases of poliomyelitis.

Note Added in Proof. A type 1/3 hybrid virus similar to but not identical with that described here was obtained also by Burke *et al.* (36). Moreover, a hybrid virus consisting of type 1 (Mahoney) whose N-AgI was replaced with N-AgI of poliovirus type 2 (Lansing) has been constructed recently by A. Martin, C. Wychowski, M. Girard, T. Couderc, M. Tardy-Panit, and R. Crainic** and by M.G.M., J. Bradley, X.-F. Yang, E.W., E. G. Moss, and V. R. Racaniello (unpublished data). The type 1/2 hybrid virus was found to be neurovirulent in mice.

**This was reported at the ICN–UCI International Conference of Virology, "Molecular Aspects of Picornavirus Infection and Detection," Jan. 14 and 15, 1988, Newport Beach, CA.

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